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Original Article

Identification of Tumorigenic Markers in Head and Neck Cancer Patients, Exploring their Correlation with Tumor Aggressiveness

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ABSTRACT

Background: Globally, head and neck cancer (HNC) considered the sixth most severe cancer associated with multiple risk factors. Due to lack of personalized therapies and undefined biomarkers, HNC shows low overall survival rate. Long non-coding RNA (LncRNA) helps to regulate multiple gene expressions in multiple biological responses. LncRNA tumor biomarkers stimulated tumor aggressiveness, cellular proliferation and inhibit apoptosis with poor prognosis. BLACAT-1, NEAT-1, HIF-1α, Caspase-3, Caspase-9 and Telomerase were associated with metastasis, tumor staging (TNM stage) activated proliferation, invasion and migration.

Objective: The presented study aims to analyze tumorigenic biomarkers, (BLACAT-1, NEAT-1 and HIF-1α, Caspase-3, Caspase-9 and Telomerase) in development and progression of head and neck cancer. Meanwhile, to observe the correlation between these biomarkers in promoting head and neck cancer.

Methods: In this comprehensive study, EDTA blood samples (5ml each) were collected from a cohort comprising 50 head and neck cancer (HNC) patients and an additional 30 healthy controls. The primary objective was to extract RNA from these samples for a detailed analysis. The study focused on evaluating the correlation between various tumor biomarkers across different HNC tumor subtypes. This correlation was meticulously analyzed using Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR), a method known for its precision in quantifying gene expression levels.

Results: BLACAT-1 shows a significant increase expression in squamous cell carcinoma. NEAT-1, Caspase-3 and Caspase-9 are non-significant in HNC tumor types whereas, inverse correlation was observed between NEAT-1 with Caspase-9 and direct proportion between NEAT-1 with Caspase-3. However, HIF-1α represented an insignificant data among all tumor subtypes under hypoxic microenvironment.

Conclusion: Higher expression levels are suggesting potential biomarkers, therefore a gene signature can be developed for determining the severity of breast tumor; therefore, can be exploited as novel therapeutic approaches to block tumor metastasis and angiogenesis.

Keywords: Neuro-oncology, Head and Neck Cancer, long non-coding RNA (Lnc-RNA), BLACAT-1, NEAT-1, HIF-1α, Caspase-3, Caspase-9, Telomerase

INTRODUCTION

Head and neck cancer (HNC), a significant global health issue, is recognized as the sixth most common cancer worldwide. It annually accounts for approximately 350,000 deaths and diagnoses near 650,000 new cases (1). This malignancy originates in various regions, including the sinuses, tongue, gums, soft palate, throat, and encompasses the nasopharynx, oropharynx, hypopharynx, larynx, lips, and salivary glands. The major symptoms associated with HNC are neuropathic pain, often due to nerve ganglions near the skull base, difficulty swallowing (dysphagia), painful swallowing (odynophagia), ear pain (otalgia), restricted jaw opening (trismus), and coughing up blood (hemoptysis) (2). The urgency to understand HNC has led to identifying biomarkers that not only highlight the severity of the disease but also contribute to its fundamental understanding.

A key focus has been on the role of certain long non-coding RNAs (lncRNAs) and their influence in inducing HNC. Among these, Bladder Cancer Associated Transcript-1 (BLACAT-1), also known as lincRNA up-regulated mainly in bladder cancer 1 (linc-UBC1), has been observed to be upregulated in various cancers including colon adenocarcinoma (COAD), stomach adenocarcinoma (STAD), and cervical squamous cell carcinoma (4). The knockdown of BLACAT-1 was found to inhibit cell proliferation by causing cell arrest at the G0/G1 phase. Conversely, BLACAT-1 overexpression led to the degradation of miR-424 and miR-143, which in turn promoted migration and invasion abilities in squamous cell carcinoma (SCC) cells. This lncRNA targets pathways such as STAT3, Wnt/β-catenin, and p15, thereby accelerating proliferation, migration, invasion, and the epithelial-mesenchymal transition (EMT) process, while also inhibiting cell cycle arrest, apoptosis, and chemotherapy sensitivity (5).

Another critical lncRNA is the Nuclear Enriched Abundant Transcript 1 (NEAT-1), a recently discovered oncogene. NEAT-1 acts as a molecular sponge, regulating the abundance and availability of miRNAs within cells. It has been implicated in promoting proliferation, inhibiting apoptosis and cell cycle arrest, regulating blood-tumor barrier permeability, and participating in the mesenchymal-epithelial transition leading to metastasis. NEAT-1 is found both in the nucleus and cytoplasm and its presence has been positively correlated with tumor stage and metastasis, thus promoting invasion and proliferation in HNC (6).

Further, the Hypoxia-Inducible Factor-1 α subunit (HIF-1α) plays a vital role as a transcriptional factor in mediating multiple protective pathways. These pathways help in reducing oxidative mechanisms and enhance oral cancer progression in oral cancer cells (7). The role of caspases, particularly Caspase-9 and Caspase-3, in apoptosis and their relevance in HNC has also been extensively studied. Caspase-9, an apoptotic biomarker, is activated during apoptosis through the binding of cytochrome c to Apaf-1 and dATP. It is known to inhibit the caspase cascade including Caspase-8 and-7 in mitochondria, thus activating cellular apoptosis. Caspase-3, a relatively smaller protein, is stimulated by various mitochondrial events such as the release of cytochrome c, Bax translocation, and AIF release, indicating its role in both intrinsic and extrinsic apoptotic pathways (8,9,10).

Lastly, the significance of telomerase in genomic stability has been underscored, with its progressive shortening in every cell division leading to chromosomal abnormalities, including cancer. The length and activity of telomerase are crucial for the progression and development of cancer. Mutations in the telomerase reverse transcriptase (TERT) promoter lead to alterations in telomere length in multiple cancerous cells. Telomerase not only acts as a prognostic marker but also offers potential in developing effective therapies against cancer. Anti-telomerase therapeutics have shown promise in initiating apoptosis and inhibiting cell viability in tumor cells, while having a lesser effect on normal cells (11,12).

MATERIAL AND METHODS

n this study, a comprehensive approach was adopted to investigate the expression levels of various biomarkers in patients diagnosed with head and neck cancer. The sample collection constituted a critical component of the research. Blood samples, precisely 5ml of EDTA blood, were collected from a cohort of 50 patients undergoing chemotherapy at different time points. This collection was carried out at the Department of Oncology, Jinnah Hospital, Lahore. To establish a control group, 30 healthy individuals also participated, providing samples under similar conditions. These samples were then transported under controlled conditions to the Molecular Diagnostic Laboratory at FCCU for subsequent processing.

The inclusion criteria for patient participation were stringent to ensure a homogeneous study population. This included patients aged between 20 and 80 years, who had been diagnosed with head and neck cancer, and who had signed the informed consent form. Conversely, the exclusion criteria were equally rigid, disallowing participation from those who had not provided informed consent or who had been treated for cancers other than head and neck cancer.

The next phase involved RNA extraction and quality checks. RNA was extracted using the Invitrogen TRIzol reagent (Catalog #15596026, USA) following the manufacturer's instructions. To ensure the integrity and purity of RNA, its quality and quantity were assessed using a Nanodrop 2000/2000c spectrophotometer (Thermo Scientific). Criteria for RNA quality included achieving 260/280 and 260/230 ratios greater than 1.5 and the presence of clear bands on agarose gel electrophoresis. In cases where these criteria were not met, the RNA was either re-precipitated or extracted anew. Following the quality assurance, cDNA synthesis was conducted using the Thermo Scientific kit (RevertAid First Strand cDNA Synthesis Kit: Catalog #K1622, USA), adhering to the manufacturer's protocol.

Primer design and optimization were critical steps. Primers specific to each biomarker - BLACAT-1, NEAT-1, HIF-1α, Caspase-3, Caspase-9, and Telomerase- were designed using the NCBI primer designing tool. These primers underwent optimization through gradient PCR to ascertain the best annealing temperatures (Tm). Additionally, the primer sequences were validated by conducting In-Silico PCR on the UCSC Genome Browser. The details of these primers were systematically tabulated (refer to Table 1).

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The expression levels of the aforementioned mRNA biomarkers were quantified using Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR). This was performed on a CFX 96 qPCR Bio-Rad, USA, employing the ThermoFisher Scientific SYBR Green qPCR Master Mix (Catalog # 4309155, USA) as per the manufacturer's guidelines.

For the statistical analysis, Graph Pad Prism Software (version 9) was utilized. The relationship between two variables was assessed using an unpaired t-test, while for three variables, one-way ANOVA with 95% confidence intervals was employed. The results were expressed as mean ± standard deviation (SD). All experiments were conducted multiple times to ensure consistency and reproducibility. A p-value of ≤0.05 was considered statistically significant, providing a robust measure for evaluating the relationships between the variables under study.

Table 1: Sequences of Selected Primers

Gene	Primer Sequence
BLACAT-1	Forward 5'TCCTCCACTGGAAGCTACAGCC3' Reverse 5'TGGCTGACAGTGTCATCAT3'
NEAT-1	Forward 5'GCTTGGCAAGGAGACTAGG3' Reverse 5'TCCCCTCTAGTCTTGGCTC3'
HIF-1α	Forward5'GAGCTCCCAATGTCGGATT3' Reverse5'GCCACTTCGAAGTAGTGCTG3'
TELOMERASE	Forward5'ATTGTGCTCACTATGCCCCG3' Reverse5'GAGTGCAGACGCGATTAGCC3'
Caspase-9	Forward5'CAGGCCCCATATGATCGAG3' Reverse5'CTGTGTCCTCTAAGCAGGAGA3'
Caspase-3	Forward5'GCGAATCAATGGACTCTGG3' Reverse 5'GACATCTGTACCAGACCGAG3'

RESULTS

Demographics of Studying Population

In conducted study 50 patients of head and neck cancer (HNC) were enrolled with 30 healthy controls. The age range of 10% study subjects was between 18-40 years and 90% of the patients were between 40-80 years. The ratio of female to male was 1:3 with standard deviation of + 8.35 and + 9.96 respectively. The division of patients on the basis of chemotherapy cycles was as 40% (cycle 1-3) and 60% (cycle 4-6). Present study divided subjects into groups for the comparative analysis between diseases patients and healthy persons as shown in Table 2.

Table 2: Tumor assessment of Head and Neck cancer Patients

Tumor stages	Cases (%)
Stage 3	40 %
Stage 4	60%
Tumor subtypes (Groups)	
Squamous cell carcinoma (SSC)	80%
Hodgkin Lymphoma (HL)	5%
Non-Hodgkin Lymphoma (NHL)	5%
Pleomorphic Adenoma (PA)	5%
Ewing's Sarcoma (ES)	5%

BLACAT-1 Expression Relatively Increase the Proliferation Potential in SCC Patients

BLACAT-1 expression significantly increased in SCC tumor subtype as compared to control while HL, NHL, PA & ES tumor subtypes shows insignificant relation. It shows that SCC directly relate with increase proliferation potential associated with metastasis and poor prognosis as shown in Figure 1. Squamous cell carcinoma with an increase BLACAT-1 expression shows its role in elevated the tumor proliferation rate, invasion and migration as compared to other HNC tumor types which were not able to take part in enhancing tumor aggressiveness among HNC patients.

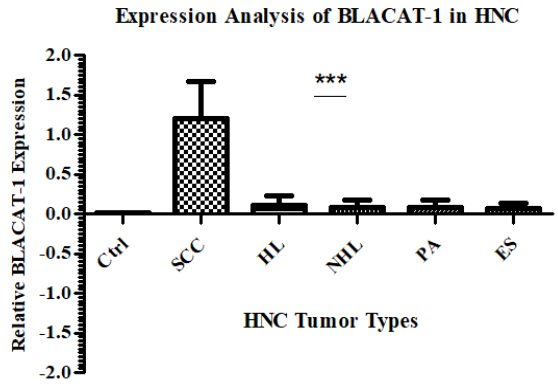

Figure 1: Expression of BLACAT-1 in HNC tunor types. Control (Ctrl), Squamous cell carcinoma (SCC), Hodgkin Lymphoma (HL), Ewing's Sarcoma (ES), Pleomorphic Adenoma (PA) & Nasopharyngeal lymphoma (NHL). * Significant difference (* $p < 0.05$; **$p < 0.01$; ***$p \leq 0.001$, not significant ($p > 0.05$).

NEAT-1 Expression Remains Unchanged in HNC Patients:

NEAT-1 expression remains insignificant in HNC tumor subtypes (SCC, HL, NHL, PA & ES) as compared to control. PA & ES shows slightly increase expression in all subtypes in comparison with control as shown in Figure 2. Pleomorphic adenoma and ewing sarcomas shows a slight response towards proliferation potential and have ability to inhibit apoptosis in comparison with other HNC tumor types and which shows no significant increase in expression after analysis.

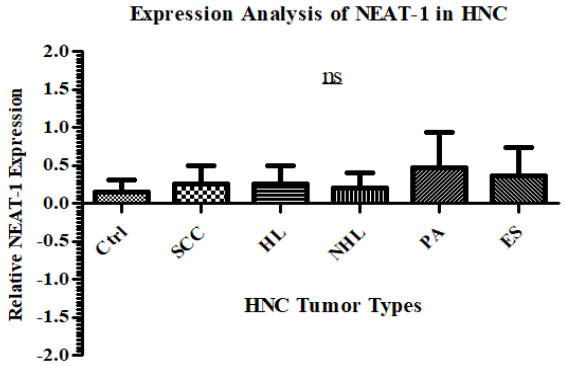

Figure 2: Expression of NEAT-1 in SCC HL, NHL, PA, & ES in HNC. Control (Ctrl), Squamous cell carcinoma (SCC), Hodgkin Lymphoma (HL), Ewing's Sarcoma (ES), Pleomorphic Adenoma (PA) & Nasopharyngeal lymphoma (NHL). * Significant difference (* $p < 0.05$; **$p < 0.01$; ***$p \leq 0.001$, ns not significant ($p > 0.05$).

Insignificance Analysis of Intrinsic Apoptotic Marker Caspase-9 HNC Patients:

Caspase-9 shows non-significant data among all the HNC tumor subtypes (SCC, HL, NHL, PA & ES) as compared to control as shown in Figure 3. It demonstrated that Caspase-9 expression did not show apoptotic activity in HNC patients. Caspase-9 an apoptotic marker shows increase expression in squamous cell carcinoma and hodgkin lymphoma as compared to other HNC subtypes. Downregulation of Caspase-9 were observed in control shows process of apoptosis were not inhibited.

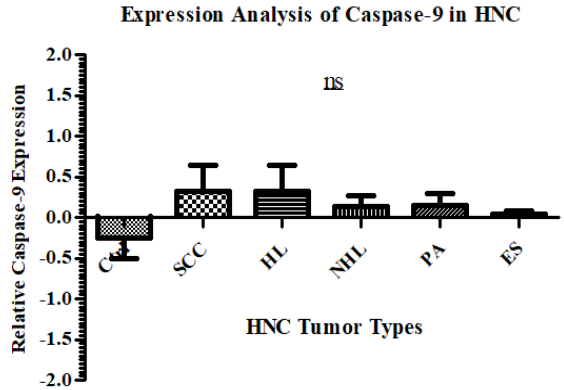

Figure 3: Expression of Caspase-9 in SCC HL, NHL, PA, & ES in HNC. Control (Ctrl), Squamous cell carcinoma (SCC), Hodgkin Lymphoma (HL), Ewing's Sarcoma (ES), Pleomorphic Adenoma (PA) & Nasopharyngeal lymphoma (NHL). * Significant difference (* $p < 0.05$; **$p < 0.01$; ***$p \leq 0.001$, ns not significant ($p > 0.05$).

Trivial Expression of Extrinsic Apoptotic Marker Caspase-3 in HNC Patients:

Insignificance analysis of Caspase-3 was observed in HNC tumor subtypes (SCC, HL, NHL, PA, ES) as compared to control. NHL shows slightly increase expression in other tumor subtypes in comparison with control shows some significance as in Figure 4. Naspharyngeal lymphoma and pleomorphic adenoma shows a slight increase expression as compared to control having ability to inhibit the process of apoptosis and activated the proliferation potential among HNC patients.

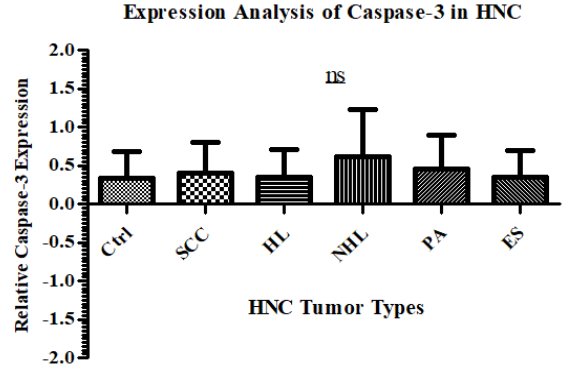

Figure 4: Expression of Caspase-3 in SCC HL, NHL, PA, & ES in HNC. Control (Ctrl), Squamous cell carcinoma (SCC), Hodgkin Lymphoma (HL), Ewing's Sarcoma (ES), Pleomorphic Adenoma (PA) & Nasopharyngeal lymphoma (NHL). * Significant difference (* $p < 0.05$; **$p < 0.01$; ***$p \leq 0.001$, ns not significant ($p > 0.05$).

Correlation of NEAT-1 with Apoptosis Marker Caspase-9 & Caspase-3:

NEAT-1 inversely corelate with intrinsic apoptotic marker such as Caspase-9 which such increase apoptosis in SCC cases as compared to HL, NHL, PA & ES tumor subtypes. NEAT-1 showed direct correlation with an extrinsic apoptotic marker such as Caspase-3. Caspase-3 expression was insignificant

in HL, NHL, PA & ES tumor subtypes as shown in Figure 5. Squamouse cell carcinoma shows a positive correlation between NEAT-1 & Caspase-3 while a negative correlation was observed between NEAT-1 and caspas-9 which demonstrated that NEAT-1 & Caspase-3 take part in increasing proliferation potential and inhibiting the cellular apoptosis (Figure 5 A). Hodgkin lymphoma expression analysis describing a direct correlation between NEAT-1 & Caspase-3 and an inverse relation was observed between Caspase-9 & NEAT-1 but no significance was observed (Figure 5 B). Nasopharyngeal lymophama demonstrated a slight change in expression between Caspase-9 & Caspase-3 while NEAT-1 did not show an increase expression illustrating that there was no correlation observed between NEAT-1 and apoptotic markers (Figure 5 C). Pleomorphic adenoma shows Caspase-3 was directly correlated with tumor marker NEAT-1 while Caspase-9 was inversely correlated with NEAT-1 expression but no trivial data was observed (Figure 5 D). Ewing's sarcomas shows a positive relation between NEAT-1 & Caspase-3 and a negation analysis was observed between NEAT-1 & Caspase-9 but again no significance was observed among them (Figure 5 E).

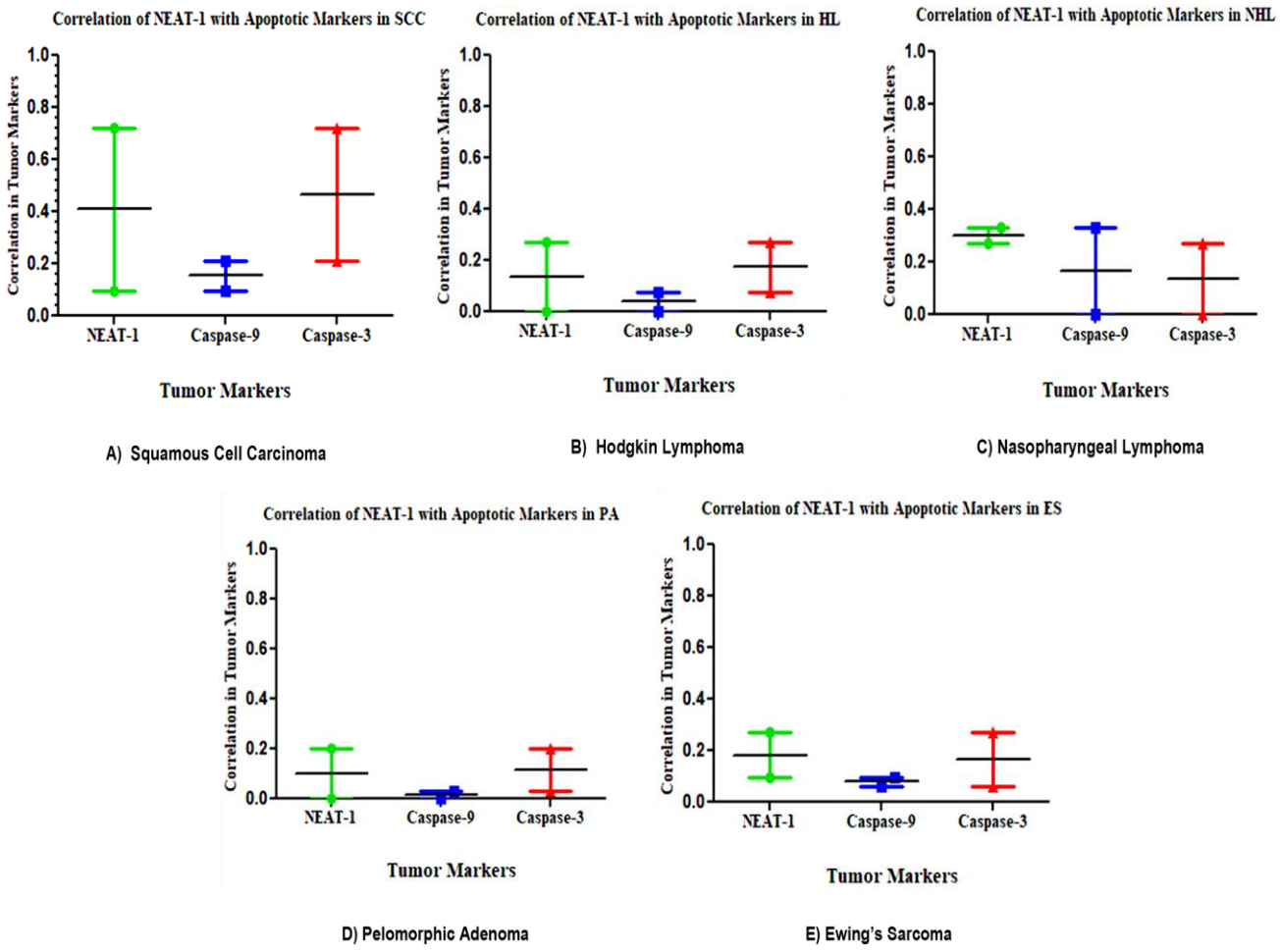

Figure 5: Correlation between NEAT-1 with Caspase-9 and Caspase-3 in HNC tumor subtypes. Control (Ctrl), Squamous cell carcinoma (SCC), Hodgkin Lymphoma (HL), Ewing's Sarcoma (ES), Pleomorphic Adenoma (PA) & Nasopharyngeal lymphoma (NHL). * Significant difference (r=1 positive correlation, r=-1 negative correlation, not significant (r=0).

Squamous Cell Carcinoma (SCC), Hodgkin Lymphoma (HL) & Ewing's Sarcomas (ES) Patients Showed Hypoxic Microenvironment: Markedly increase expression of HIF-1α as compared to control shows change in microenvironment in HL & ES patients subtype towards hypoxic whereas SCC, NHL & PA shows no significant change in HIF-1α levels. HIF-1α level remains slightly increased in SCC, NHL, PA subtypes but insignificant as compared to control as shown in Figure 6. Hodgkin lymphoma, ewing's sarcoma & squamous cell carcinoma expression analysis demonstrated that they have ability to proliferate under hypoxic environment as compared to the other tumor subtypes of HNC patients.

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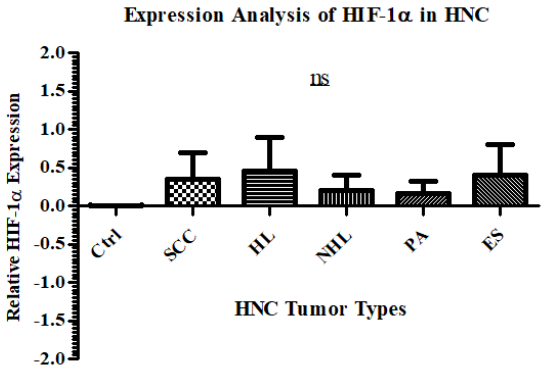

Figure 6: Expression of HIF-1α in SCC HL, NHL, PA, & ES in HNC. Control (Ctrl), Squamous cell carcinoma (SCC), Hodgkin Lymphoma (HL), Ewing's Sarcoma (ES), Pleomorphic Adenoma (PA) & Nasopharyngeal lymphoma (NHL). Significance was determined by one-way ANOVA. * Significant difference (* $p < 0.05$; ** $p < 0.01$; *** $p \leq 0.001$, ns not significant ($p > 0.05$).

Insignificant Role of Telomerase in Development of Tumor Environment Modulation:

Telomerase expression levels remain trivial in SCC, HL, NHL, PA & ES tumor subtypes. It shows that a slightly elevated expression was observed in PA as compared with the control but no significance was determined as in Figure 7. Pleomorphic adenoma represented a slightly increase expression as compared to other tumor subtypes shows change in telomeric length while other tumor types did not show any significant change in telomerase length.

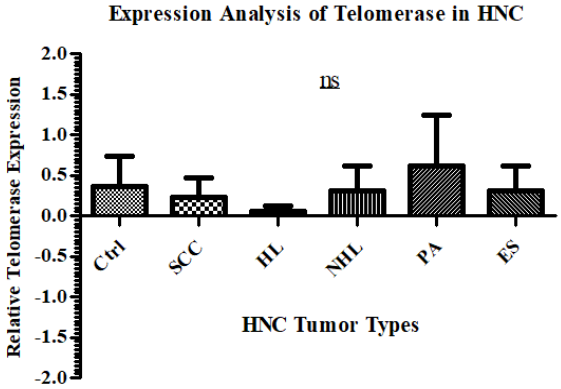

Figure 7: Expression of Telomerase in SCC HL, NHL, PA, & ES in HNC. Control (Ctrl), Squamous cell carcinoma (SCC), Hodgkin Lymphoma (HL), Ewing's Sarcoma (ES), Pleomorphic Adenoma (PA) & Nasopharyngeal lymphoma (NHL). * Significant difference (* $p < 0.05$; ** $p < 0.01$; *** $p \leq 0.001$, ns not significant ($p > 0.05$).

No significant association between tumor stage and genes expression:

Majority of patients of stage-3 disease shows intrinsic apoptotic potential whereas the disease prognosis of stage-4 intrinsic apoptosis take overs. Significantly elevated expression of BLACAT-1 was seen in advanced stage (stage-4) patients. NEAT-1 and HIF-1α expression did not show any relationship with the severity of disease and remains stable. In stage-3 patients Caspase-3 level was higher as compared to Caspase-9 showing intrinsic apoptotic modulation.

DISCUSSION

The study presented a comprehensive analysis of the expression levels of various biomarkers in head and neck cancer (HNC) patients, providing insights into the complex mechanisms of tumor development and progression. One of the key findings was the significant role of Bladder Cancer Associated Transcript-1 (BLACAT-1) in the pathogenesis of squamous cell carcinoma (SCC). The expression levels of BLACAT-1 were markedly increased in SCC patients compared to other tumor subtypes and were notably higher in advanced tumor stages (Stage-4). This observation aligns with previous studies that highlighted the importance of BLACAT-1 in promoting cancerous mechanisms such as migration and invasion (13). Furthermore, an inverse relationship was identified between BLACAT-1 and miR-142-5p expression, suggesting that BLACAT-1 overexpression leads to the inhibition of miR-142-5p and consequently activates oral SCC (OSCC) (14). The downregulation of BLACAT-1 resulted in increased cellular apoptosis and reduced cellular viability, inducing cell cycle arrest. This finding supports the notion that BLACAT-1 is intricately linked with cellular proliferation and apoptosis in HNSCC, and its low expression suppresses migration and invasion in oesophageal SCC (ESCC), highlighting its potential as a therapeutic target (15).

In contrast, NEAT-1 expression showed a negative correlation with the intrinsic apoptotic marker Caspase-9, with insignificant expression observed across all tumor subtypes. However, a direct relationship was noted with the extrinsic pathway marker Caspase-3 in all HNC subtypes, indicating a complex interaction between NEAT-1 and apoptotic pathways in HNC. Despite these findings, no significant data emerged between NEAT-1 and Caspase-3 across all tumor subtypes (17). The study's findings on NEAT-1 are in line with previous research that demonstrated its association with poor pathological factors and its role in inhibiting angiogenesis and cellular proliferation in ESCC by regulating the MDM2-p53 pathway (18).

The activation of caspases, particularly Caspase-9 and Caspase-3, was another focus area. Limited work has been done on caspase activation in HNC patients alone, but existing literature suggests their crucial role in inducing apoptosis and inhibiting cellular proliferation in malignant tumors. For instance, the activation of these caspases during arsenic trioxide (As2O3) treatment has been reported to induce apoptosis and inhibit proliferation in malignant tumors (19). This study's findings suggest a potential inverse correlation between Caspase-9 and As2O3 expression in HNSCC, along with the implication that Caspase-3 activation, through

pathways involving Caspase-9 or Apaf-1, might have anti-apoptotic effects in inducing tumorigenesis and poor prognosis in HNC, thereby serving as a potential tumor biomarker (21).

In terms of the study's limitations, it is important to acknowledge the relatively small sample size and the focus on a specific geographical region, which may limit the generalizability of the findings. Additionally, while the study provided valuable insights into the role of various biomarkers in HNC, the complex interactions between these markers and other cellular pathways warrant further investigation. Moreover, the study primarily focused on genetic and molecular aspects, potentially overlooking other relevant factors such as environmental and lifestyle influences on HNC progression.

The study also explored the role of Hypoxia-Inducible Factor-1α (HIF-1α) in HNC. It was observed that decreased oxygen levels did not significantly increase the proliferation rate among HNC patients. This finding is consistent with a meta-analysis that demonstrated an increase in HIF-1α C1772T & G1709A polymorphism associated with increased metastasis in HNC patients. However, this study also highlighted that smaller HL cells required more HIF-1α expression compared to larger cells in biopsied samples of lymph nodes (23). The role of HIF-1α in modulating the hypoxic microenvironment and its impact on mitochondrial activity via PDK-1 in immature HL cells further underscores its significance in HNC pathology.

Lastly, the study examined the changes in telomere length and their impact on HNC tumor subtypes. While the expression of Pleomorphic adenoma (PA) was slightly increased compared to the control group, no significant data was determined. The expression level of telomerase reverse transcriptase (TERT) promoter was elevated in cervical SCC (CSCC) with mutated TERT regions compared to non-mutated OSCC. This finding suggests a potential role of HPV16 E6 in TERT expression (24). In vitro studies have shown that increased expression of the TERT promoter region inversely correlates with therapeutic efficacy in HNSCC (25,26), indicating that elevated levels of TERT transcript may serve as a prominent prognostic marker in cancer development and progression in HNSCC.

This study contributes to the growing body of research on the molecular mechanisms underlying HNC. The findings underscore the importance of exploring the role of various biomarkers in the development and progression of HNC and highlight potential therapeutic targets. Future research should aim to expand upon these findings, considering a larger and more diverse patient population and exploring the interplay of genetic, environmental, and lifestyle factors in HNC progression.

CONCLUSION

The current study offers a pioneering direction in the analysis of biomarkers, shedding light on the intricate biological responses elicited by tumor cells in head and neck cancer (HNC). This research underscores the potential of these biomarkers in advancing therapeutic strategies. One of the critical findings is that an expanded sample size, particularly with well-defined time intervals during chemotherapy, could substantially improve the robustness and efficacy of the results. It emphasizes the need for further research involving larger sample sizes, starting from the baseline in HNC patients and tracking changes through each chemotherapy cycle. Such an approach would enable a more detailed and dynamic understanding of the variations in various parameters, thereby enhancing the overall effectiveness of the findings.

Moreover, the study suggests that a more comprehensive analysis of gene expressions is required. Utilizing a variety of techniques to examine these expressions could yield more significant data, providing stronger evidence for disease prognosis. This comprehensive approach to gene expression analysis is crucial for developing a more nuanced understanding of the disease and its progression. Ultimately, the findings from this study pave the way for more targeted and effective therapeutic interventions in HNC, with the potential to significantly improve patient outcomes.

ETHICAL STATEMENT

This study was conducted after the approval of Ethical Review Committee (ERC-74-2021) and Institutional Review Board (IRB-305/07-2021) of Forman Christian College (A Charted University) along with approval of Ethical Review Board, Jinnah Hospital Lahore (94/08/06-2021-S2.ERB).

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